Minimizing Skeletal Muscle Injury to Ischemia/Reperfusion with Adenosine A_3 Receptor Agonists: Role of Matrix Metalloproteases

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ABSTRACT

Introduction

Today's Warfighter is subject to traumatic injuries to skeletal muscle. Tourniquet use for hemorrhage control is common, as well as in surgical situations requiring the restriction of blood flow to an extremity. The reperfusion period following prolonged tourniquet use results in skeletal muscle damage, making ischemia/reperfusion (I/R) injury a concern for military personnel. In regards to acute injury, 63% of injuries sustained in combat are the result of explosive munitions that produce traumatic injury in skeletal muscle.

Rationale

Work from our laboratory has determined that Adenosine A_3 receptors are a novel therapeutic target in attenuating I/R injury in skeletal muscle. Administration of A_3 receptor agonists prior to I/R resulted in a striking reduction in gastrocnemius muscle injury in mice. The data on A_3 protection in skeletal muscle provide important evidence of a cytoprotective role of the adenosine A_3 receptor. How adenosine A_3 receptors act to reduce skeletal muscle injury is not well understood. Given the importance of metallothioneins (MTs) and matrix metalloproteases (MMPs) in skeletal muscle remodelling, questions arise as to whether A_3 receptor intervention would modulate the MT and MMP response, subsequently minimizing skeletal muscle damage and facilitating a more rapid recovery/return to duty. The purpose of this work was to extend previous studies to elucidate the mechanisms associated with A_3 agonist muscle protection and to define the role of the MT/ MMP pathway.

Methods

Mice were pre-treated with the A_3 receptor agonist or a vehicle 2 h prior to ischemia. Ligation of the hindlimb was performed in all mice for 2 h followed by 24 h of reperfusion and tissue collection. Evans blue dye staining, which quantifies the number of injured muscle cells, and serum creatine kinase (CK) levels were used to assess muscle damage. qRT-PCR, immunoblotting, and zymography were used to quantify the effect of I/R injury on transcription, translation, and activity, respectively, of the MTs and MMPs.

Results

Adenosine A_3 receptor agonist pre-treatment reduced skeletal muscle injury with a significant 20 % decrease in Evans blue dye staining and an 85% decrease in serum CK. A_3 receptor agonist pre-treatment

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14. ABSTRACT
Todays Warfighter is subject to traumatic injuries to skeletal muscle. Tourniquet use for hemorrhage control is common, as well as in surgical situations requiring the restriction of blood flow to an extremity. The reperfusion period following prolonged tourniquet use results in skeletal muscle damage, making ischemia/reperfusion (I/R) injury a concern for military personnel. In regards to acute injury, 63% of injuries sustained in combat are the result of explosive munitions that produce traumatic injury in skeletal muscle.

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also increased transcription of MT mRNA and reduced MMP mRNA following injury. The anti-inflammatory molecule MMP-9 increased with A_3 receptor agonist treatment.

Conclusion

A_3 receptor agonist pre-treatment may be an important intervention to minimize skeletal muscle damage in response to I/R injury. I/R injury results in significant increases in transcription of the MTs and MMPs, and treatment provides protection at the transcriptional level within 24 h of injury. It will be important to determine if A_3 receptor agonists can also mitigate muscle damage associated with other types of muscle injury such as blunt trauma, burn or cold stress. Development of an A_3 receptor agonist intervention that could be administered in the acute phase of injury may be an important means of reducing traumatic muscle injury in the operational setting.

1.0 INTRODUCTION

Sixty-three percent of injuries sustained by today's Warfighter are the result of explosive munitions (Scarborough 2007). The force of these explosions and fragmented debris from the device itself and nearby structures produce traumatic injury to skeletal muscle and surrounding tissues. Tourniquet use for hemorrhage control is mandatory in theater, as well as in surgical situations that require the restriction of blood flow to an extremity. Often, upon removal of the tourniquet, the subsequent reperfusion of blood to previously ischemic tissue induces inflammation, morphological abnormalities and tissue necrosis (Carmo-Araujo et al. 2007). As the most vulnerable tissue in the extremities, ischemia/reperfusion (I/R) can cause significant injury to skeletal muscle, exacerbating pre-existing injury, resulting in loss of function, delayed healing, and incomplete rehabilitation. Prolonged hospitalization as a result of delayed recovery of skeletal muscle function and integrity severely impacts the number of active duty and reserve personnel available for combat and military missions.

Thus, protection of skeletal muscle from I/R injury is an important therapeutic goal directed toward ameliorating muscle and organ injury in military populations. Although various measures such as a tissue-preserving solutions and cold immersion are used to preserve intact organs and skeletal muscle (Southard and Belzer 1995; Tsuchida et al. 2003), an effective method or agent to protect skeletal muscle from I/R injury is lacking. Recent data have demonstrated that adenosine receptor agonists and ischemic preconditioning can provide potent protection of the heart muscle when administered prior to a myocardial infarction (Mozzicato et al. 2004). As a result, interest is emerging to study whether manipulation of adenosine receptors can also induce protection of skeletal muscle.

1.1 The Role of Metallothioneins and Matrix Metalloproteases

Metallothioneins (MTs) are small (12-14 kDa), ubiquitous, cysteine-rich, zinc-binding proteins which are primarily produced in the liver and released into the circulation (Tapiero and Tew 2003). Upon release into the circulation metallothioneins play a pivotal role in cellular processes that render protection to all tissues of the body. In skeletal muscle, MTs initiate anti-inflammatory and anti-apoptotic signalling cascades, reduce reactive oxygen species (ROS)-induced cytotoxicity, protect against ROS-induced DNA degradation, and maintain zinc homeostasis (Feng et al. 2006; Tapiero and Tew 2003). Marked induction of MT mRNA is evident in skeletal muscle of animals and humans under conditions that promote oxidative stress such as I/R and traumatic injury (Kondo et al. 1992; Lecker et al. 2004; Penkowa et al. 2005).

While the specific role of MT is to neutralize ROS, MMPs contribute directly to tissue remodelling in both healthy and pathological muscle (Birkedal-Hansen et al. 2008). MMPs process extracellular matrix proteins, cytokines and growth factors, and optimal remodelling of the extracellular matrix is contingent on tightly regulated MMP activity (Kjaer 2004). Induction of the MMPs is largely dependent on the

substrate affected, as specific MMPs are activated to degrade collagens (MMP-1, -8, -13, and -18), gelatins (MMP-2, and -9), stromelysins (MMP-3, -10, and -11), and membrane-type proteins (MMP-14, -15, -16, and -17).

Several lines of evidence suggest an involvement of ROS in the cascade of events initiating skeletal muscle remodelling, particularly following I/R injury when skeletal muscle cells are more susceptible to oxidative stress (Jagoe et al. 2002; Lecker et al. 2004; Warren et al. 2007). Concomitant increases in the expression of MT and metalloproteases (MMPs) has been reported in response to skeletal muscle injury and during the remodelling phase (Lecker et al. 2004; Warren et al. 2007). These findings imply that the signalling cascade connecting injury, I/R, the release of ROS, MT induction, and MMP-induced remodelling is a prime candidate for pharmacological intervention.

Pharmacological attenuation of MMP induction and downstream proteolytic cascades, or stimulation of MMP inhibitors such as TIMP-1 and TIMP-2, has the potential to prevent additional injury, while reducing the time to recovery post-trauma (Hnia et al. 2007). Moreover, pharmacological agents designed to reduce ROS-induced membrane damage by enhancing antioxidant molecules such as MT, may mitigate the increase in proteolytic signalling following injury in skeletal muscle.

1.2 Adenosine A_3 Receptor Agonists: Mechanism of Action

Adenosine A_3 receptors were recently identified as a novel therapeutic target in attenuating I/R injury in cardiac muscle (Zheng et al. 2007). Thus, the A_3 receptor agonist is a prime candidate for pharmacological intervention in skeletal muscle based on several working hypotheses regarding its mechanism of protection in response to injury. First, previously published data suggest that the activation of A_3 receptors is capable of inducing potent anti-ischemic protection of skeletal muscle (Zheng et al. 2007). Potentially, A_3 receptor activation can induce a greater induction of MT following injury, providing the muscle with a greater antioxidant defense system subsequently suppressing oxidant-induced proteolytic signalling cascades. A second working hypothesis regarding the mechanism of protection by the A_3 receptor agonist involves its anti-inflammatory properties. The primary event linking skeletal muscle injury to intracellular proteolytic events is the infiltration of inflammatory cells in the hours and days post-injury. It has been suggested that this inflammatory reaction may produce additional damage, increasing the possibility for muscle fibrosis, scarring, and subsequent injury (Tidball 1995).Thus, limiting certain aspects of inflammation through A_3 receptor modulation may reduce muscle degeneration as well as signalling mechanisms for muscle scarring (Sicard 2002). Finally, the role of the A_3 receptor agonist in mitigating Ca^{2+} influx and overload, through its effect on Phosphokinase-C (PKC) signalling, has the potential to decrease the activation of MMPs and the subsequent increase in proteolytic cascades. Essentially, the mechanism for this protective response involves the injury-induced disturbances in Ca^{2+} homeostasis resulting in elevated intracellular Ca^{2+}. This increase in cellular Ca^{2+}activates the cysteine protease calpain which plays a critical role in triggering skeletal muscle protein breakdown, inflammatory changes, and regenerative processes (Inserte et al. 2006; Stracher 1999). Figure 1 illustrates how these proposed mechanisms are involved in reducing skeletal muscle injury following A_3 receptor agonist treatment.

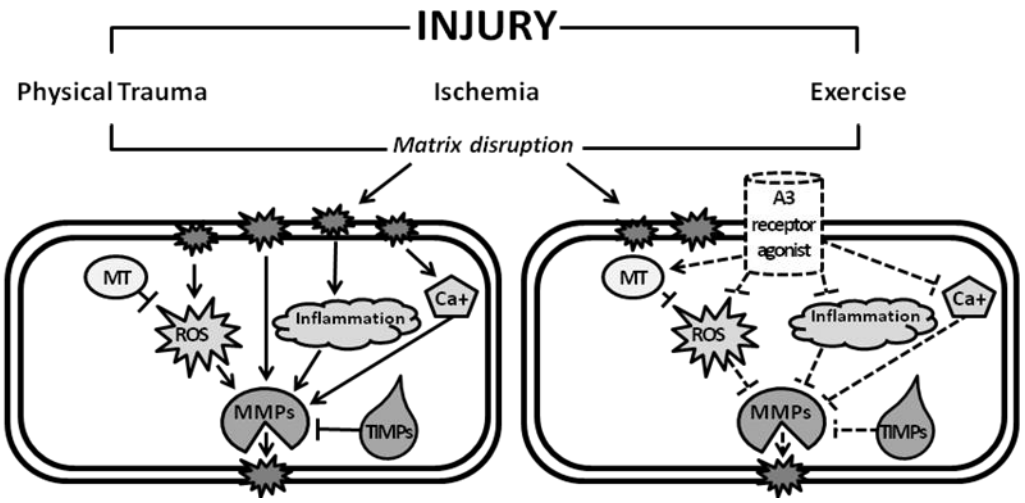

Figure 1: Model of adenosine A_3 receptor signalling mechanism in cytoprotection of skeletal muscle: Working model of the relationship between skeletal muscle injury, intracellular signalling, and potential modes of A_3 receptor activation on these pathways. The left panel depicts the natural pathology associated with skeletal muscle injury (solid lines). The right panel illustrates the proposed signalling pathways affected by A_3 receptor agonist treatment (dashed lines). Arrows represent actions that promote the activity of downstream molecules, while flat lines indicate mitigation or inhibition. MMP-matrix metalloproteinase, MT-metallothionein, ROS-reactive oxygen species, TIMP-tissue inhibitor of metalloproteinase.

Based on these working hypotheses, we investigated the effects of pre-treatment with the A_3 receptor agonist in skeletal muscle of mice following 2 h of ischemia. Our goal was to characterize the effect of A_3 receptor agonist treatment on transcription and translation of MT, MMPs, and TIMPs, as well as markers of skeletal muscle damage 24 h post-reperfusion.

2.0 METHODS

Twenty (N=20: 10 A_3 Group, 10 Placebo Group), three-month old, wild-type C57BL6 mice, weighing ~23-25g were anesthetized with phenobarbitol sodium (50 mg/kg/ip). Mice received two injections 2 h prior to the start of ischemia. The first was an ip injection (0.07 mg/kg) of the A_3 receptor agonist (Cl-IBMECA) or a placebo (PBS) in DMSO. The second was the Evans Blue Dye (1% wt/vol solution, 1mg EBD/10g body wt) in PBS. Two hours after the injection, the right hindlimbs of the mice were elevated to minimize retained blood and ligation was performed using a constrictor band placed above the greater trochanter using a McGivney Hemorroidal Ligator (7 in long, Miltex). Following 2 h of ischemia (37C) the constrictor was removed and the limb reperfused for 24 h. Twenty-four hours post-reperfusion, serum was collected from a tail vein for creatine kinase (CK) analysis. Mice were then given an anesthetic overdose, and the gastrocnemius muscles of the injured and uninjured leg were harvested and snap frozen in liquid nitrogen. The gastrocnemius was used because of its high proportion of fast twitch muscle, which is highly prone to I/R injury. Evans blue dye staining (EBD), a dye which is taken up by muscle cells that have been injured, and serum CK levels were used to quantify skeletal muscle injury. Quantitative real time polymerase chain reaction (qRT-PCR), immunoblotting, and zymography were used to quantify the effect of I/R injury on transcription, translation, and activity, respectively, of MT and the MMPs. All data from I/R legs were normalized to data from the uninjured leg in the same animal. Statistical analysis was conducted using the SPSS statistical package (v.13.0, SPSS Inc., Chicago, IL). An ANOVA followed by a Tukey's post-hoc was used to analyze the statistical significance of mRNA, protein, EBD and CK data between the A_3 and Placebo Groups. Alpha was set at 0.05. All data are presented as means ± SE.

3.0 RESULTS

3.1 Markers of Muscle Injury

A_3 receptor agonist treatment protected against CK release and resulted in a significant reduction in serum CK. Serum CK levels in A_3-treated mice were 1,840 ± 910 U/L versus 12,600 ± 3,300 U/L in the Placebo Group ($P<0.05$). Histochemical analysis of EBD staining compliments CK data, demonstrating a reduction in skeletal muscle injury post-I/R in A_3-treated mice versus those in the Placebo Group. Average EBD staining of skeletal muscle sections following I/R injury and A_3- or Placebo- treatment were quantified in the uninjured and injured legs. The uninjured leg, which was not subjected to I/R, showed no EBD staining. A_3-treated mice had a significant, 20% decrease in the percent of EBD positive cells as compared to Placebo-treated mice. In the A_3-treated mice, EBD staining was evident in 5.4 ± 2.6% of the analyzed cells, while in the Placebo-Group, EBD staining was present in 28.0 ± 6% of the analyzed cells, confirming the efficacy of the A_3 treatment in protecting skeletal muscle cells from I/R injury (Figure 2).

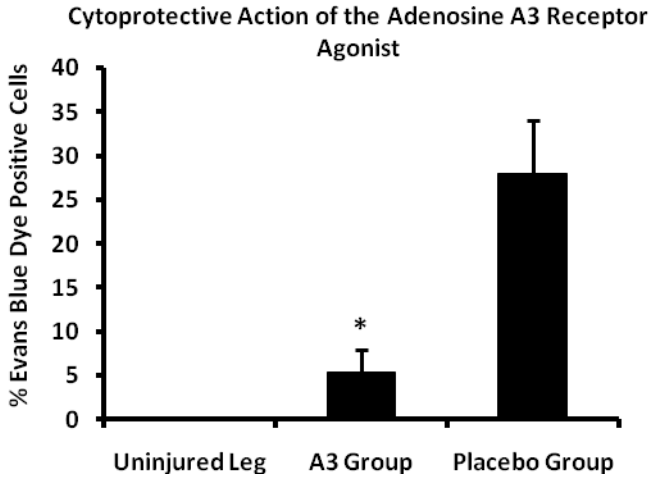

Figure 2: Cytoprotective action of Adenosine A_3 receptor agonist treatment in skeletal muscle: Evans Blue Dye (EBD), a stain which is taken up by injured cells, was absent in uninjured leg muscles from the A_3-treated and Placebo Groups. In the I/R injured leg, the number of positive cells for EBD staining was significantly reduced in the inured leg of the A_3-treated Group as compared to the Placebo Group (*$p<0.05$).

3.2 MT mRNA and Protein

Following I/R injury, MT mRNA expression in the Placebo Group was significantly reduced 2.3-fold in the injured leg. Treatment with the A_3 receptor agonist promoted a 26- fold increase in MT mRNA in the injured leg versus the uninjured leg. Interestingly, despite this robust difference in mRNA, while protein levels of MT increased approximately 87% in the injured leg, there were no differences in MT protein levels between the A_3 Group and the Placebo Group. These robust increases in mRNA were not accompanied by changes in metallothionein protein levels in the A_3 or Placebo Group (Figure 3).

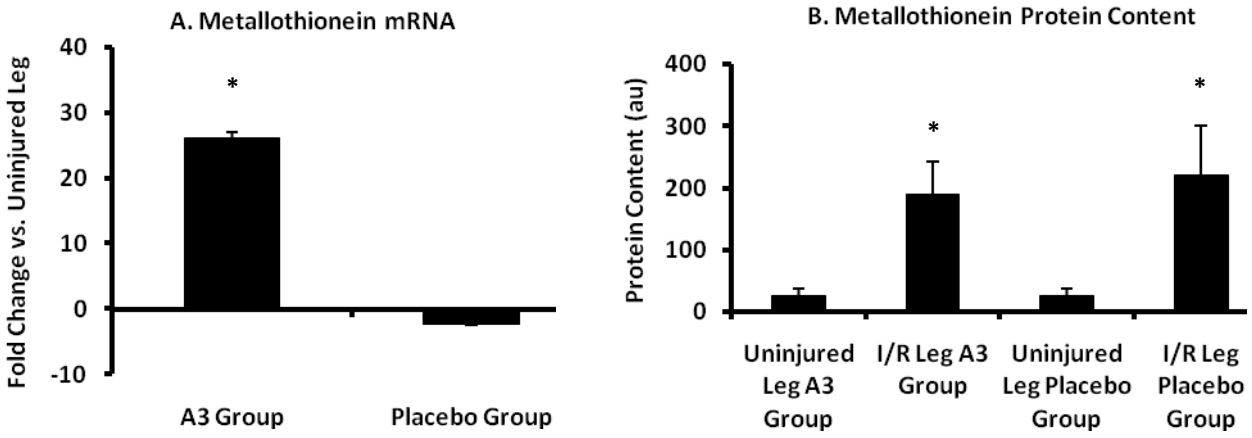

Figure 3: Metallothionein mRNA and Protein Expression: A. mRNA expression of Metallothionein increased significantly in the I/R leg of the A_3-treated mice. mRNA expression in the I/R leg was significantly higher in the A_3 Group versus the Placebo Group (*p<0.05). B. Metallothionein protein content was significantly higher in I/R injured leg versus the uninjured leg, regardless of treatment group. *p<0.05 vs. Uninjured Leg.

3.3 MMP and TIMP mRNA and Protein Levels

Our data show that transcription of MMPs is significantly inhibited by A_3 receptor agonist treatment. MMP-2 expression increased 18.4- fold in the I/R leg of the Placebo Group, while A_3 treatment blunted this increase resulting in a modest, 4.5-fold increase in MMP-2 mRNA in the I/R leg ($p<0.05$). Similar results were seen for MMP-3 and MMP-14 with mRNA levels increasing 11.9- and 51.8-fold, respectively, in the I/R leg in the Placebo Group ($p<0.05$). With A_3 receptor agonist treatment, however, MMP-3 and MMP-14 mRNA levels were only upregulated 1.8- and 16.0- fold, respectively in the I/R leg versus the uninjured leg ($p<0.05$). Interestingly, MMP-9 mRNA was decreased 1.9- fold in the I/R leg of the Placebo group, but significantly upregulated 5.6- fold in the I/R leg of the A_3 Group ($p<0.05$).

TIMP-1 and TIMP-2, the inhibitors of the metalloproteases, were also evaluated to understand their response to I/R injury and the effect of A_3 treatment on mRNA expression. Data indicate that pre-treatment with the A_3 receptor agonist promotes enhanced MMP inhibition, with TIMP-1 mRNA expression increasing 9.1-fold in the I/R leg of the A_3 Group, significantly higher than the 3.9-fold increase observed in the Placebo Group (Figure 4). There were no significant differences in TIMP-2 expression between Groups, with TIMP-2 expression levels increasing approximately 2- fold in the I/R leg, regardless of treatment (Figure 4).

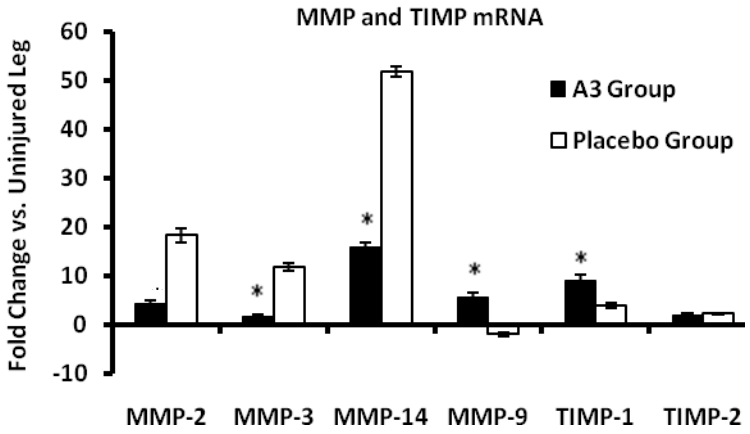

Figure 4: mRNA Expression of the MMPs and their inhibitors, the TIMPs: mRNA expression of MMP-2, -3, and -14 was significantly lower in the I/R leg of the A_3 Group versus the Placebo Group (*p<0.05). MMP-9, which possesses anti-inflammatory properties, exhibited increased mRNA expression in the I/R leg of the A_3 Group versus the Placebo group. TIMP-1 mRNA activity was increased in the A_3 Group, suggesting enhanced protection in the A_3 Group. *p<0.05 vs. Placebo Group.

There were no significant differences in protein levels of the MMPs (-2, -3, -9, and -14) or the TIMPs (-1 and -2) in the I/R leg versus the uninjured leg 24 h post-reperfusion. Thus, at this time point post-reperfusion, there were no detectable differences in protein levels as a result of A_3 receptor agonist treatment. Gelatin zymography was also performed to assess MMP-2, -3, and -9 activity. In contrast to western blotting results, MMP-3 activity was increased in the I/R leg as compared to the uninjured leg, with no significant differences in MMP-3 activity in the I/R leg between the A_3 and Placebo Groups.

4.0 DISCUSSION AND CONCLUSIONS

The data presented here indicate that A_3 receptor agonists play an important role in modulating the MT, MMP and TIMP response in skeletal muscle. Work from our laboratories confirms that mRNA alterations in MT, the MMPs and the TIMPs are truly robust in response to I/R injury and A_3 receptor agonist treatment affects transcriptional activity of these molecules. Indeed, we did not detect differences in protein levels in response to A_3 agonist treatment. Thus, we cannot conclude with absolute certainty that A_3 agonist treatment will provide protective benefits in skeletal muscle following I/R injury. However, based on skeletal muscle translational efficiency, it is possible that A_3-induced alterations in the protein levels of MT, MMPs, and TIMPs may be modulated several days post-injury, rather than within the first 24 h when the muscle is reaching a new steady state. Overall, these preliminary data suggest that pharmacological activation of the adenosine A_3 receptor may modulate MT, MMPs and TIMPs in skeletal muscle following I/R injury, providing benefit to skeletal muscle in regards to remodelling and regeneration.

For example, our observed increase in MT post-I/R injury in the group receiving the A_3 receptor agonist suggests that treatment renders protection to skeletal muscle against inflammation, apoptosis and ROS (Feng et al. 2006; Tapiero and Tew 2003). Marked induction of MT mRNA post-I/R injury in the treated animals is thought to counteract the infiltration of inflammatory molecules and the upregulation of proteolytic cascades which is common post-I/R injury. Reduction of inflammatory molecules and ROS is particularly important in promoting regeneration in skeletal muscle, as excessive infiltration of inflammatory molecules combined with ROS-induced proteolysis delays healing and results in an overall loss in skeletal muscle tissue. In some cases when this response is extreme, healthy tissue is also degraded in addition to damaged tissue. Instances like these critically impact the rate and degree of healing post-injury. Ultimately, the integrity of tissue subjected to robust increases in inflammatory molecules is compromised and more susceptible to future injury (Lecker et al. 2004; Penkowa et al. 2005; Scheede-Bergdahl et al. 2005). Thus, improving the MT

response in skeletal muscle post-injury is paramount and A_3 receptor treatment appears to be a promising countermeasure to regulate MT induction in skeletal muscle.

Similarly, our work demonstrates that A_3 receptor agonist treatment manipulates MMP and TIMP transcription in skeletal post-I/R injury. Indeed, MMPs contribute to skeletal muscle tissue remodelling through extracellular matrix degradation and repair (Birkedal-Hansen et al. 2008), however, the presence of exceedingly high levels of MMP mRNA and protein has led to the suggestion that in addition to degrading damaged tissue post-injury, the MMPs degrade healthy, strength-giving extracellular matrix components. Therefore, A_3 receptor agonist treatment, which we have shown reduces the expression of MMPs within the first 24 h post-injury, may increase the stability of regenerating skeletal muscle tissue. Additionally, MMP-9 which was the sole MMP demonstrating an increase in expression as a result of A_3 receptor treatment, serves an important role as an anti-inflammatory molecule when released in injured tissues (Kjaer 2004; Ogawa et al. 2005; Rossignol et al. 2007). Thus, A_3 treatment-induced increases in the expression of MMP-9 may further minimize inflammatory cascades that lead to additional tissue destruction and necrosis. Collectively, this response accelerates the time to recovery.

Finally, although modest, our use of A_3 agonist treatment resulted in an increase in mRNA expression of TIMP-1 following I/R injury. This increase is important as TIMP-1 is a natural inhibitor of the MMPs (Gomis-Ruth et al. 1997; Lluri and Jaworski 2005). Furthermore, in addition to its inhibitory role against the MMPs, TIMP-1 promotes cell proliferation and has anti-apoptotic functions in skeletal muscle. Therefore, through manipulation of TIMP-1 expression via A_3 receptor agonist treatment, skeletal muscle is further protected against apoptosis of healthy cells. Additionally, migration of quiescent cells necessary for regeneration, such as satellite cells, is facilitated.

In summary, our work demonstrates the efficacy of A_3 receptor agonist treatment in protecting skeletal muscle from I/R injury within the first 24 h post-injury. We have demonstrated that A_3 receptor agonist treatment can manipulate MT, MMPs, and TIMPs, groups of molecules critical for initiating antioxidant defences and skeletal muscle remodelling, respectively, post-injury. The results of our work presented here emphasize the need for additional research that focuses on the efficacy of A_3 receptor agonists: in the days post-injury; on skeletal muscle function; and in response to various military-relevant injuries such as burn, trauma, and overuse injury. We are currently conducting research to establish the efficacy of A_3 receptor agonist treatment on MT, MMP, and TIMP expression in skeletal muscle post-traumatic injury using a model that mimics blunt-force injury sustained by troops in theatre. This work focuses on the acute and chronic efficacy of A_3 treatment in manipulating proteolytic pathways, as well as the implications of treatment in preserving skeletal muscle function and expediting the time to recovery post-injury.

5.0 DISCLAIMER

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. Any citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement of approval of the products or services of these organizations. In conducting the research described in this report, the investigators adhered to the "Guide for Care and Use of Laboratory Animals" as prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

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